IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Mitani et al.	Examiner:	Mummert, Stephanie
Serial No.:	10/583706	Group Art Unit:	1637
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Title:	METHOD OF AMPLIFYING NUCLEIC ACID AND METHOD OF DETECTING MUTATED NUCLEIC ACID USING THE SAME		

DECLARATION UNDER 37 CFR §1.132

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Commissioner for Patents
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Dear Sir:

I, Takefumi Ishidao, hereby declare as follows:

1. I graduated from
1996, March; Department of Biological Science, Faculty of Science, Kumamoto University.
1998, March; Master's Course, Graduate School of Science and Technology, Kumamoto University.
2001, March; Doctor's Course, Department of Biological Sciences, Graduate School of Science, and Faculty of Science Osaka University.

2. I have worked in
2001, April; Department of Molecular and Genetic Information, Institute of Molecular and Cellular Biosciences, Tokyo University
2004, April; Laboratory of Molecular Genetics, RIKEN Tsukuba Institute
2007, September; Kabushiki Kaisya DNAFORM.

3. I consider myself to be an expert in the field of molecular biology, knockout mice, cancer research and cell division.

4. Under my direction, the following experiments were conducted.

Experiment

1. Experiment Objective

Using (i) TP + FP (present invention), (ii) TP + TP, and (iii) FP + FP as primer pairs, the amplification effects of respective primer pairs were compared.

2. Experiment Description

2. 1. Confirmatory Experiment 1

In this example, it was attempted to amplify the human STS DYS237 gene using Human Genomic DNA (manufactured by Promega) as a template. The sequences and the properties of the primers used for the experiments are described below. Furthermore, the positional relationship of each primer region to the template was set as illustrated in FIG. 1 and the following sequences. The following sequences are partial sequences of the template. The single-underlined part in the following sequences represents a region to which the 3' end regions of respective forward primers are annealed and the double-underlined part in the following sequence represents a region to which the 3' end region of the reverse primer is annealed.

1 aagcttttaa agcatcctca tttaigtcc aacatcagag acttaatact gaacaaatgc cacataaagg taatgactgt

81 tgaagaagat ttaacttaac atcttgcagc atcactaaga actcgcttta tactcagtgc tttgggttg ggtttg

A forward primer F1 (FP) is designed so as to have the structure shown in FIG. 2 in which the sequence (22 mer: the single-underlined part) that is located on its 3' end side anneals to the template, while the sequence (16 mer: the part other than the single-underlined part) that is located on the 5' end side is folded in that region.

F1: 5'- ggatatatatatccactgaacaaatgccacataaag -3'

A forward primer F2 (TP) is designed so that the sequence (22 mer: the single-underlined part) that is located on its 3' end side anneals to the template, while after an extension reaction, the sequence (10 mer: the part other than the single-underlined part) that is located on the 5' end side hybridizes to the region starting from 27 bases downstream of residues located at the 3' end of the primer on the strand extended by the primer.

F2: 5'- aagatgttaaactgaacaaatgccacataaag -3'

A forward primer F3 (TP) is designed so that the sequence (22 mer: the single-underlined part) that is located on its 3' end side anneals to the template, while after an extension reaction, the sequence (10 mer: the part other than the single-underlined part) that is located on the 5' end side hybridizes to the region starting from 25 bases downstream of residues located at the 3' end of the primer on the strand extended by the primer.

F3: 5`- gatgttaagtactgaacaaatgccacataaag -3`

A reverse primer R1 (TP) is designed so that the sequence (20 mer: the double-underlined part) that is located on its 3' end side anneals to the template, while after an extension reaction, the sequence (10 mer: the part other than the double-underlined part) that is located on the 5' end side hybridizes to the region starting from 16 bases downstream of residues located at the 3' end of the primer on the strand extended by the primer.

R1: 5`- gcagcatcaccaacccaaaagcactgagta -3'

The aforementioned primers were used as the primer pairs described below.

Primer pair 1 (FP + TP)

F1: 5`- ggatatatatatccactgaacaaatgccacataaag -3'

R1: 5`- gcagcatcaccaacccaaaagcactgagta -3'

Primer pair 2 (TP + TP)

F2: 5`- aagatgttaaactgaacaaatgccacataaag -3'

R1: 5`- gcagcatcaccaacccaaaagcactgagta -3'

Primer pair 3 (TP + TP)

F3: 5`- gatgttaagtactgaacaaatgccacataaag -3'

R1: 5`- gcagcatcaccaacccaaaagcactgagta -3'

(1) Amplification Experiment

A reaction solution (25 μL) having the following composition was prepared: Tris-HCl (20 mM, pH 8.8), KCl (10 mM), $(NH_4)_2SO_4$ (10 mM), $MgSO_4$ (8 mM), DMSO (3%), Triton X-100 (1%), dNTP (1.4 mM), 2000 nM of each primer of the aforementioned primer pairs, a template (100 ng), and 16U Bst DNA polymerase (NEW ENGLAND BioLabs). This was incubated at 60°C for 60 minutes or for 65 minutes. The template was allowed to react while being maintained in the double-stranded state. As a control, the same experiment was carried out with respect to a solution in which sterile water was added instead of the template.

With respect to each reaction solution 5 μL, electrophoresis was carried out at 100 V for 40 minutes using 4% NuSieve 3:1 Agarose (manufactured by BioWhittaker Molecular Applications (BMA) Inc.; purchased from TAKARA BIO INC.; "NuSieve" is a registered trademark of BMA Inc.). After the electrophoresis, the gel thus obtained was stained with ethidium bromide (EtBr) and thereby nucleic acids were detected. The results are shown in FIGs. 3 and 4. FIG. 3 shows the result of the experiment in which the reaction solution was incubated for 60 minutes. FIG. 4 shows the result of the experiment in which the reaction solution was incubated for 65 minutes. The samples in the respective lanes shown in FIGs. 3 and 4 are as follows:

Lane 1: 20 bp DNA Ladder size marker

Lane 2: a reaction solution in which the primer pair 1 and a template were added

Lane 3: a reaction solution in which the primer pair 1 and sterile water instead of a template were added

Lane 4: a reaction solution in which the primer pair 2 and a template were added

Lane 5: a reaction solution in which the primer pair 2 and sterile water instead of a template were added

Lane 6: a reaction solution in which the primer pair 3 and a template were added

Lane 7: a reaction solution in which the primer pair 3 and sterile water instead of a template were added

As a result of the incubation for 60 minutes, as shown in FIG. 3, no bands were observed in Lanes 3, 5, and 7, i.e., solutions (controls) in which sterile water was added instead of the template. Further, no amplification product was observed in Lane 4, i.e., a solution in which the primer pair 2 (TP + TP) was used and the template was added. In contrast, amplification products were observed in Lane 2, i.e., a solution in which the primer pair 1 (FP + TP) was used and the template was added, and Lane 6, i.e., a solution in which the primer pair 3 (TP + TP) was used and the template was added. Specifically, a ladder-like electrophoresis result was obtained in each of the Lanes 2 and 6.

Further, as a result of the incubation for 65 minutes, as shown in FIG. 4, amplification products were observed in Lanes 2, 4, and 6. Specifically, a ladder-like electrophoresis result was obtained in each of the Lanes 2, 4, and 6. As in the case of FIG. 3, no bands were observed in Lanes 3, 5, and 7 each serving as a control.

(2) Cleavage by Restriction Enzyme

In order to prove that the amplification products obtained in the (1) amplification experiment were derived from the target nucleic acid sequence, the amplification products were digested with a restriction enzyme. Specifically, 0.3 μL of the reaction solution obtained after the amplification reaction in the (1) amplification experiment was digested (at 37°C for 3 hours) with a restriction enzyme MboII.

The digestion product was electrophoresed using 4% NuSieve 3:1 Agarose (manufactured by BioWhittaker Molecular Applications (BMA) Inc.; purchased from TAKARA BIO INC.; "NuSieve" is a registered trademark of BMA Inc.). The results are shown in FIG. 5. The samples in the respective lanes shown in FIG. 5 are as follows:

Lane 1: 20 bp DNA Ladder size marker

Lane 5: 20 bp DNA Ladder size marker

Lane 2: a digestion product of 0.3-μL amplification product subjected to incubation for 60 minutes using the primer pair 1

Lane 3: a digestion product of 0.3-μL amplification product subjected to incubation for 60 minutes using the primer pair 2

Lane 4: a digestion product of 0.3-μL amplification product subjected to incubation for 60 minutes using the primer pair 3

Lane 6: a digestion product of 0.3-μL amplification product subjected to incubation for 65 minutes using the primer pair 1

Lane 7: a digestion product of 0.3-μL amplification product subjected to incubation for 65 minutes using the primer pair 2

Lane 8: a digestion product of 0.3-μL amplification product subjected to incubation for 65 minutes using the primer pair 3

As shown in FIG. 5, since the bands of the undigested sample were changed into the bands with predicted sizes after the digestion with a restriction enzyme, it was proved that the target nucleic acid sequence had been amplified.

2. 2. Confirmatory Experiment 2

In this example, using pPACKH1-GAG (manufactured by System Biosciences) as a template, it was attempted to amplify HIV gag gene contained therein. The sequences and the properties of the primers used for the experiments are described below. Furthermore, the positional relationship of each primer region to the template was set as illustrated in FIG. 6 and the following sequences. The following sequences are partial sequences of the template. The single-underlined part in the following sequences represents a region to which the 3' end region of the forward primer A1 is annealed and the double-underlined part in the following sequence represents a region to which the 3' end region of the reverse primer A2 is annealed.

1 gatggatgacaaataatccacctatcccagtaggagaaatttataaaagatggataatcctgggattaa

71 ataaaatagtaagaatgtatagccctaccagcattctggacataagacaaggaccaaaaga

A forward primer A1 is designed so as to have the structure shown in FIG. 7 in which the sequence (28 mer: the single-underlined part) that is located on its 3' end side anneals to the template, while the sequence (52 mer: the part other than the single-underlined part) that is located on the 5' end side is folded in that region.

A1: 5'- agactttacatcaagcatactggtgctaaccagtatgcttgatgtaaagtctataatccacctatcccagtaggagaaat -3'

A reverse primer A2 is designed so as to have the structure shown in FIG. 8 in which the sequence (28 mer: the double-underlined part) that is located on its 3' end side anneals to the

template, while the sequence (52 mer: the part other than the double-underlined part) that is located on the 5' end side is folded in that region.

A2: 5'- cgattaatcacttcttaagactgcgctagcagtcttaagaagtgattaatcgtttggtccttgtcttatgtccagaatgc - 3'

(1) Amplification Experiment

A reaction solution (25 μL) having the following composition was prepared: Tris-HCl (20 mM, pH 8.3), KCl (25 mM), $MgCl_2$ (1.5 mM), Tween (0.05%), dNTP (50 μM), autoclaved gelatin (100 μg/mL), 200 pmol of each primer of the aforementioned primer pairs, a template (1×10^5 to 1×10^6 copy), and 3U Taq DNA polymerase (NEW ENGLAND BioLabs) or 6U Aac DNA polymerase. This was incubated at 95°C for 1 minute, then cooled, and then incubated at 63°C for 5 minutes for carrying out annealing and extension of the forward primer A1 with respect to each strand. Further, the second incubation was carried out at 95°C for 1 minute, and then the reaction solution was incubated at 63°C for 120 minutes. Since Aac DNA polymerase does not have super thermal stability, it was added again after the second incubation of at 95°C for 1 minute.

Further, in order to have suitable conditions for Aac DNA polymerase, an Aac DNA polymerase optimum reaction solution (25 μL) having the following composition was prepared: Tris-HCl (20 mM, pH 8.8), KCl (10 mM), $(NH_4)_2SO_4$ (10 mM), $MgSO_4$ (8 mM), DMSO (3%), Triton X-100 (1%), dNTP (1.4 mM), 200 pmol of each primer of the aforementioned primer pairs, and a template (1×10^5 to 1×10^6 copy). This was incubated at 95°C for 1 minute. Then, since Aac DNA polymerase does not have super thermal stability, the reaction solution was cooled and then 6U Aac DNA polymerase was added. Thereafter, the reaction solution was incubated at 63°C for 5 minutes for carrying out annealing and extension of the forward primer A1 with respect to each strand. Further, after the second incubation was carried out at 95°C for 1 minute, 6U Aac DNA polymerase was added again and the reaction solution was incubated at 63°C for 120 minutes.

With respect to each reaction solution 5 μL, electrophoresis was carried out at 100 V for 40 minutes using 0.8% Agarose (manufactured by Invitrogen Corporation). After the electrophoresis, the gel thus obtained was stained with ethidium bromide (EtBr) and thereby nucleic acids were detected. The results are shown in FIG. 9. The samples in the respective lanes shown in FIG. 9 are as follows:

Lane 1: 1 Kbp DNA Ladder size marker

Lane 2: a reaction solution in which Taq DNA polymerase and a template were added

Lane: 3 a reaction solution in which Aac DNA polymerase and a template were added

Lane 4: a reaction solution having optimum conditions for Aac DNA polymerase in which Aac DNA polymerase and a template were added

No high-molecular weight (50 kb or more) amplification product described in Exhibit No. 3 was observed in any of the lanes.

[FIG. 1]

1 aagcttttaa agcatcctca ttttatgtcc aacatcagag acttaatact gaacaaatgc cacataaagg taatgactgt

3' end region of F primer

81 tgaagaagat ttaacttaac atcttgcagc atcactaaga actcgcttta tactcagtgc ttttgggttg ggtttg

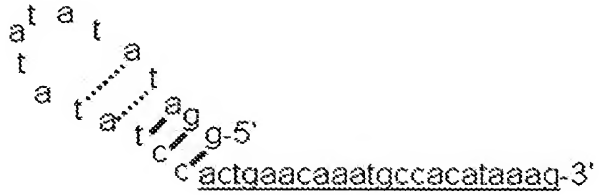
folded part of R1 primer

3' end region of R1 primer

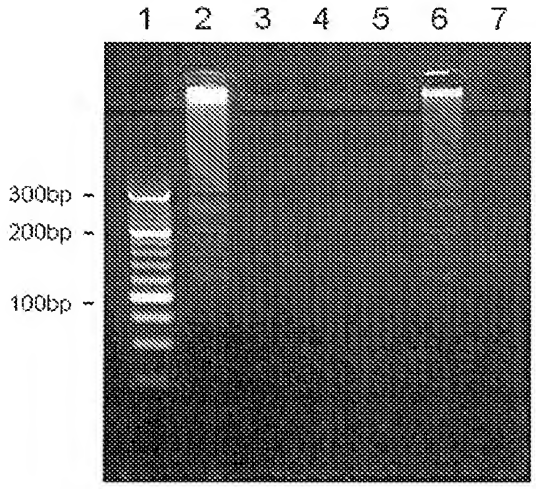
folded part of F2 primer

folded part of F3 primer

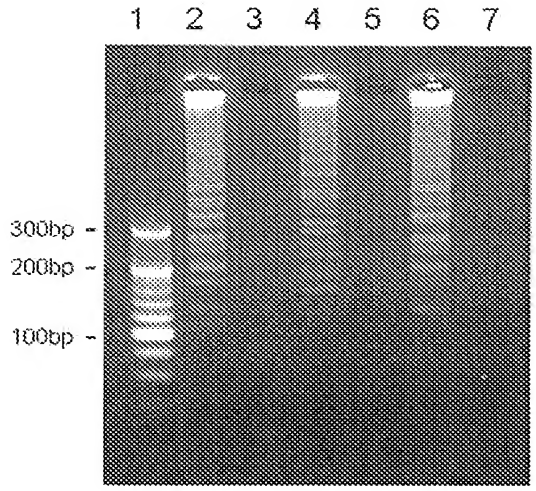
[FIG. 2]



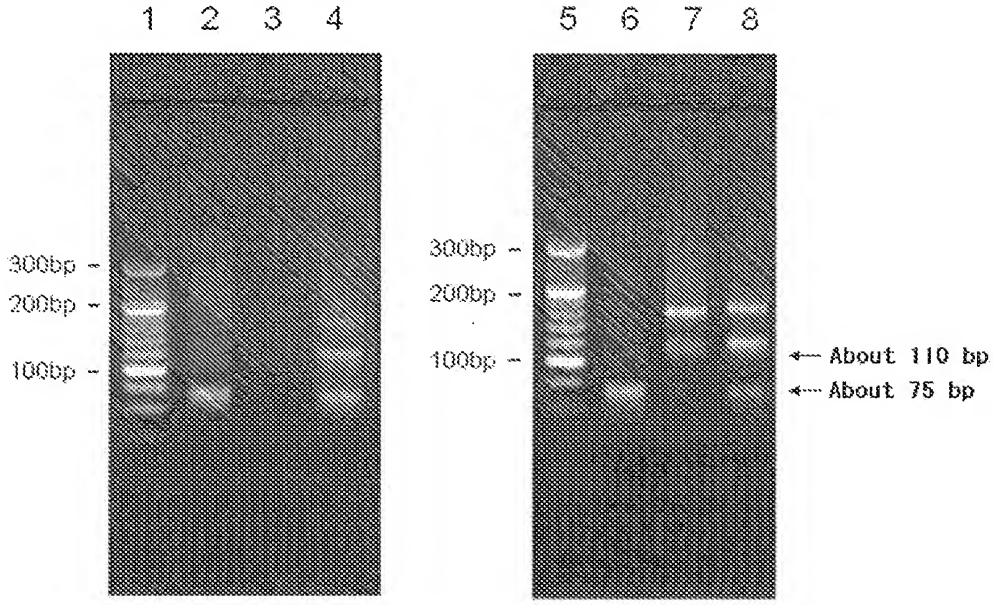
[FIG. 3]



[FIG. 4]


1 2 3 4 5 6 7
300bp
200bp
100bp

[FIG. 5]


1 2 3 4
5 6 7 8
300bp
200bp
100bp
300bp
200bp
100bp
About 110 bp
About 75 bp

[FIG. 6]

1 gatggatgacaaataatccacctatcccagtaggagaaatttataaaagatggataatcctgggattaa

3' end region of A1 primer

71 ataaaatagtaagaatgtatagccctaccagcattctggacataagacaaggaccaaaaaga

3' end region of A2 primer

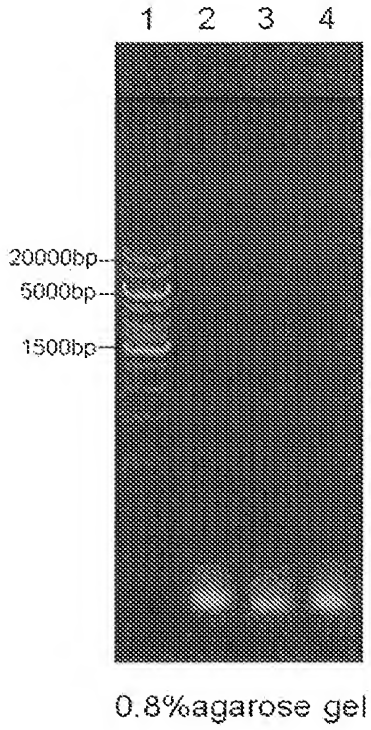
[FIG. 7]

c g tggtcatacgaactacatttcaga-5'
t a accagtatgcttgatgtaaagtct ataatccacctatcccagtaggagaaat-3'

[FIG. 8]

c g cgtcagaattcttcactaattagc
t a gcagtcttaagaagtgattaatcg tttggtccttgtcttatgtccagaatgc-3'

[FIG. 9]



0.8%agarose gel

I declare under the penalty of perjury of the laws of the United States of America that the foregoing is true and correct to the best of my information and belief.

Signed this 5 of Feb 2012, at Yokohama, JAPAN

Takefumi Ishidao

Takefumi ISHIDAO